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PHYSICOCHEMICAL STUDIES OF CHLOROPLAST STRUCTURAL PROTEIN AND ^{14}C-LEUCINE INCORPORATION INTO CHLOROPLAST PROTEINS

by

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ABSTRACT

Chloroplasts from wheat and bean seedlings were isolated using sucrose-tris and sucrose-phosphate buffers. Wheat chloroplasts were purified by repeated washings while bean chloroplasts were purified on a discontinuous sucrose density gradient. After removing the lipid and pigments the protein was solubilized in cholate and deoxycholate. The structural protein fraction was isolated by ammonium sulfate fractionation. Sedimentation velocity studies showed homogeneous profiles in both species with S values lower than that reported for spinach. Concentration dependence studies revealed no association or dissociation of the protein. The amino acid composition of this protein fraction from bean and wheat was similar. Optical rotatory dispersion measurements indicated the presence of α-helix in both. Gel electrophoresis showed that with respect to charge the structural protein fraction of wheat and bean chloroplasts was different and not homogeneneous.

When wheat leaves excised from 3.5, 4.5 and 5.5 day old seedlings were fed with uniformly labeled ^{14}C-L-leucine, label was incorporated into the chloroplast proteins. Chloroplasts were purified on a discontinuous sucrose density gradient prior to separation of the soluble and the particulate proteins. Distribution of the label between the soluble and the particulate protein changed with age. Chase experiments were carried out to determine if the soluble proteins served as precursors of particulate

proteins. Seventy-five minutes and 18 hours chase experiments were carried out with leaves from 4.5 day old seedlings in light. Neither the 75 minutes nor 18 hours chase altered the distribution of the label between the soluble and the particulate proteins relative to the 90 minutes feeding.

Following 18 hours chase in light with leaves from 3.5 day old seedlings, there was a change in the distribution of the label. Eighteen hours chase experiments conducted in the dark revealed that the membrane proteins were relatively more stable than the soluble proteins. Significant radioactivity was found in the chloroplast structural protein fraction.

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Symbols and Abbreviations used

ATP	Adenosine 5'-triphosphate
CTP	Cytidine 5'-triphosphate
DTT	Dithiothreitol
GTP	Guanosine 5'-triphospate
tricine	Hydroxymethyl methylglycine
tris	Hydroxymethyl aminomethane
ORD	Optical rotatory dispersion
PEP	Phosphoenolpyruvate
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
S	Svedberg unit
UTP	Uridine 5'-triphospate
$[\alpha]_{\lambda}$	Specific rotation
λ_c	Dispersion constant

INTRODUCTION

Recent years have seen major advances in our knowledge of chloroplast chemistry and function. Chloroplasts were initially interesting for biologists because of their photosynthetic function. However, photosynthesis is only one of many exciting biological capacities of the chloroplast. Recent findings concerning the ability of isolated chloroplasts to synthesise lipids and proteins have provided new and fascinating problems to the plant biochemist.

In order to understand the various functions of chloroplasts, it is important to know the nature of their chemical constituents. The chemistry of the chloroplast proteins is largely unknown although they represent more than one third of the chloroplast components. In chloroplasts the particulate proteins constitute as much as 80% of the proteins. According to Criddle (1966) structural protein amounted to 50% of the particulate proteins in spinach chloroplasts. Although structural protein represents about 40% of total chloroplast proteins, only very limited information about them is available and this is partly due to the difficulties in their isolation and solubilization. Criddle and Park (1964) isolated this protein fraction from spinach chloroplasts by using surface active agents and ammonium sulfate fractionation. In bean and wheat the chloroplast structural protein fraction represented about 40% of the particulate proteins. Thus this study was undertaken to characterize and compare chloroplast

structural protein fractions from bean and wheat by sedimentation velocity studies, optical rotatory dispersion measurements, amino acid analysis and polyacrylamide gel electrophoresis.

In recent years isolated chloroplasts have been shown to incorporate labeled amino acids into protein. The label appeared in both, the soluble and the particulate proteins. Since the structural protein fraction accounted for nearly 50% of the particulate proteins, it might be expected to become labeled. Hence ^{14}C-leucine incorporation experiments with excised leaves from wheat seedlings were carried out. It was anticipated that studies involving incorporation of label into chloroplast proteins would elucidate the sequence of incorporation and the degree of stability of the structural protein fraction, which might help in defining its structural role.

LITERATURE REVIEW

I. Chloroplast Proteins

Chloroplasts are the major protein bearing bodies of the leaf. Chloroplasts of tobacco and bean leaves for which comparative studies were made were found to contain about 75% of the total leaf nitrogen (Stocking and Ongun, 1962). The total protein content of spinach chloroplasts which were isolated in aqueous media was 50% on dry weight basis and 69% when a non-aqueous isolation procedure was employed (Kirk and Tilney-Bassett 1967). Chloroplast proteins may be classified into soluble and insoluble or particulate proteins. The particulate proteins are associated with lamellar fragments which are obtained upon centrifugation of ruptured chloroplasts. The supernatant contain the soluble proteins.

Wildman and Bonner (1947) isolated two soluble protein fractions from spinach leaves. Of these the faster moving and major component in the analytical ultracentrifuge was designated as fraction I protein, while the slower moving component was called fraction II protein. Fraction I protein was homogeneous in the analytical ultracentrifuge while the slower moving component was heterogeneous. Lyttleton and Ts'o (1958) and Heber *et al.* (1963) showed these protein components to be associated with the chloroplasts. Fraction I protein was later shown to have ribulose diphosphate carboxylase activity (Mayaudon *et al.* (1957) and Lyttleton and Ts'o (1958). Weissbach *et al.* (1956) and Trown (1965)

showed that the physical properties of this enzyme and the fraction I protein were also similar. It is now generally agreed that fraction I protein is ribulose diphosphate carboxylase.

Fraction I protein had a sedimentation coefficient of 18s (Wildman and Bonner, 1947; Pon, 1967). It was an acidic protein with an isoelectric point around pH 4.3 - 5.5. Fraction I protein from oat leaves was essentially homogeneous by gel electrophoresis in tris-glycine buffer pH 8.4 Steer et al. (1966). Later Pon (1967) showed the fraction I protein from spinach chloroplasts was also homogeneous by moving boundary electrophoresis. As it constituted about 70 to 80% of chloroplast soluble protein, additional functions were considered for it (Park and Pon, 1961; Kupke, 1962). According to Van Noort et al., (1961) and Mendiola and Akazawa (1964) it also had ribose phosphate isomerase and phosphoribulokinase activities. However Thornber et al. (1965) showed that the isomerase and kinase activities could be separated from fraction I protein by column chromatography. Trown (1965) obtained fraction I protein with no isomerase or kinase activities and he explained the other enzyme activities in fraction I protein as being due to their adsorption on carboxydismutase during isolation. Fraction I protein from Chinese cabbage leaves under the electron microscope appeared as cubical particles with the side of the cube being 120A° long (Haselkorn et al. 1965). Akazawa et al. (1965) demonstrated the glycoprotein nature of fraction I protein preparations from rice leaves.

Lamellar Proteins

Proteins associated with the chloroplast membranes are termed as lamellar proteins. Since the whole architecture of the chloroplast involves intricate membrane systems the proteins associated with these membranes should play an important role in the structure and organization of chloroplasts. Thus far, however, only little progress has been made in understanding the chemistry of these proteins. This has been due largely to the difficulties in isolation and solubilization of these proteins.

Menke and Jordan (1959) were the first to study in detail this protein which they referred to as chloroplast lamellar structural protein. In their study they took advantage of the stability, that is the insoluble character of this fraction in neutral aqueous buffers. They obtained lamellar structural protein from chloroplasts after removing lipids and pigments by extraction with methanol and ether and the soluble proteins were removed by extraction with dilute NaCl and NaOH. Lamellar structural protein so obtained accounted for roughly 50% of the total weight of the chloroplast preparation.

Weber (1962) obtained lamellar proteins from four different chloroplast sources (*Chlorella pyrenoidosa*, *Alliumporrum Spinacia oleracea* and *Antirrhinum majus*). Lockshin and Burris (1966) isolated lamellar proteins from chard and corn chloroplasts using butanol extraction. The amino acid composition of all these proteins was remarkably similar. Weber (1963) prepared a soluble

lamellar structural formylated protein from chloroplasts of *Antirrhinum majus*. The formylated protein had a sedimentation coefficient of 5.9S and its amino acid composition differed only slightly from that of Menke and Jordan (1959).

According to Menke and Jordan (1959) and Weber (1962, 1963) the lamellar structural protein constituted all the residual components after extraction of chloroplast fragments with organic solvents, dilute aqueous salt and dilute sodium hydroxide. Biggins and Park (1965) and Ji et al. (1968) isolated lamellar protein with an S value of 2.2 from spinach chloroplasts. Ji et al. (1968) showed it to be heterogeneous by disc-gel electrophoresis in borate buffer pH 8.5. The amino acid composition was similar to that of spinach chloroplast lamellar protein as reported by Bailey et al. (1966), Criddle and Park (1964) and Weber (1962). Molchanov and Bezinger (1968) isolated lamellar protein with an S value of 2.9 from bean chloroplast and 1.7 from corn. Bean chloroplast lamellar protein was heterogeneous by polyacrylamide gel electrophoresis while that from corn was homogeneous.

Structural Protein

Much of the present understanding of the chemical nature of chloroplasts has been drawn from the extensive studies on mitochondria. The basis of this has been the remarkable similarity between the two organelles in certain features.

Green et al.(1961) isolated a protein fraction from solubilized beef heart mitochondrial membranes by ammonium

sulfate fractionation which they designated as mitochondrial structural protein. Since then several methods have been proposed for its isolation (Richardson et al.1964; MacLennan, 1965; Woodward and Munkres, 1966 and Lenaz et al.1968). A recent review of these methods has been published by Criddle (1969). Structural protein accounted for roughly 40% of the total protein of mitochondria and had a molecular weight of 23,000. On the other hand Blair et al. (1968) have shown the molecular weights to range from 55,000 to 70,000. However, it was homogeneous by analytical ultracentrigufation with an S value of 0.95.

Criddle and Park (1964) isolated by a procedure similar to that used for mitochondria a protein fraction from spinach chloroplast membranes. The protein fraction was obtained by ammonium sulfate precipitation in the region of 12-16%. They designated this membrane protein fraction as chloroplast structural protein and according to them it constituted roughly 50% of the total chloroplast lamellar protein. It had an S value of 2.2 and was homogeneous in analytical ultracentrifuge analysis and by moving boundary electrophoresis. Its amino acid composition was similar to that of spinach chloroplast lamellar protein (Weber 1962). However, the structural protein was significantly higher in the levels of aspartate, glutamate and lysine and notably lower in phenylalanine when compared with spinach chloroplast lamellar protein. The protein fraction obtained by Menke and Jordan (1959 is generally referred to as chloroplast lamellar protein and the protein fraction

obtained by Criddle is referred to as chloroplast structural protein. In fact all membrane proteins are structural proteins, as they all contribute in some way to structure and stability. But of these some may be more tightly associated with the membrane than others. There is no basis of selecting a unique protein component to which a more significant structural role can be ascribed. His reason for calling this particular protein fraction structural protein was based on the fact that it accounted for roughly 50% of the total membrane protein and he could detect no other function for it, and also by analogy with other membrane systems which have structural proteins (mitochondria, microsomes and membrane proteins from red blood cells) Richardson et al. (1963).

Since its isolation, physical and chemical properties of the structural protein fraction have been studied with a view to elucidate its structural role. For a protein to play a structural role it should be fairly stable and this criterion is readily met by its insolubility in neutral aqueous buffer. However, the chloroplast structural protein could be dissolved in a solution containing 0.5m urea and 0.1% sodium dodecyl sulfate at pH values greater than 10.5. Also for a protein to play a structural role, it should be able to interact with the other components of chloroplasts namely lipids, pigments and cytochromes. Criddle and Park (1964) showed binding of these with the chloroplast structural protein. These results are in agreement with the concept of the role of structural protein, and from these one may conclude that this protein fraction

should play an important role in the structure of membrane systems.

Lenaz et al. (1968) demonstrated the heterogeneity of mitochondrial structural protein by gel electrophoresis in acidic medium. Gel electrophoretic studies of chloroplast structural protein from bean and wheat form a part of this thesis. The structural protein fraction from both species was heterogeneous (Mani and Zalik 1970).

Optical rotatory dispersion measurements have been used to distinguish membrane systems from soluble proteins and polypeptides. In membrane systems the cotton effect showed a red shift of 2 to 3 mμ (Lenard and Singer 1966). This effect was observed with, chloroplast fragments (Ke 1965), *Bacillus subtilis* membrane (Lenard and Singer 1966) and mitochondrial vesicles (Urry et al. 1967). Solubilized mitochondrial structural protein had an ORD spectrum characteristic of a protein containing α-helical structure and aggregation resulted in a spectral shift (Steim and Fleischer 1967). According to Steim and Fleischer, the spectral shift was due to structural protein interaction within the membrane. However this spectral shift should not be taken as a specific property of interacting membrane proteins, as it was also shown upon aggregation of the α-helical poly-L-glutamic acid (Cassim and Yang 1967).

In addition to soluble and lamellar proteins only a few other chloroplast proteins had been studied in detail. Davenport and Hill (1952) isolated cytochrome f from parsley leaves

(Petroselinum sativum). They estimated it to have a molecular weight of 110,000 on the basis of sedimentation and diffusion measurements. Its isoelectric point was at pH 4.7 and it had 2 haem groups per molecule. Cytochrome f from *Euglena gracilis* had a molecular weight of 17,000 and an isoelectric point at pH 5.5. Although it is a known constituent of chloroplasts, cytochrome b_6 has not been purified as yet. Katoh and Takamiya (1963) isolated a copper containing protein from chloroplast called plastocyanin. It is an acidic protein with a molecular weight of 21,000. Ferredoxin, the iron-containing non-haem protein of chloroplast with a molecular weight of 12,200 was isolated by Bendall et al. (1963). Packer and Marchant (1964) and Ohnishi (1964) have isolated a protein fraction from chloroplasts which underwent conformational changes with ATP. They considered it as a contractile protein on analogy with the results obtained with muscle and on the basis of the isolation procedure which was similar to that employed for actomyosin from muscle.

Chloroplast ribosomal proteins have been studied with a view to comparing them with the surrounding cytoplasmic ribosomal proteins. Biswas et al. (1968), Lyttleton (1968) and Odintsova and Yurina (1969) found a difference between the chloroplast and the cytoplasmic ribosomal proteins of pea and spinach when compared by electrophoresis. Although Lyttleton (1968) obtained 2 bands which were characteristic of cytoplasmic ribosomal proteins from 8 different sources, neither of these bands was present in spinach

chloroplast ribosomal protein. It should be interesting to see whether one gets a characteristic protein pattern in the case of chloroplast also.

II. Protein Synthesis by Chloroplasts

As indicated earlier in the literature review, protein is in terms of quantity the major chloroplast component. It was therefore expected that chloroplasts were capable of protein synthesis and considerable study has been directed along this line. Several reviews have appeared on the mechanism of protein synthesis in cytoplasm (Hoagland 1960; Gale 1962; Arnstein 1965). If chloroplasts synthesise proteins, they must possess all the components essential for protein synthesis. Chloroplasts have been shown to contain RNA (Brawerman 1963; Brawerman and Eisenstadt, 1964; Sissakian *et al*. 1965, Hadziyev *et al*. 1968) DNA (Chun *et al*. 1963; Sager and Ishida, 1963; Ray and Hanawalt, 1964; Brawerman and Eisenstadt, 1964; Kung and Williams, 1968, 1969), ribosomes (Lyttleton, 1960, 1962; Clark *et al*. 1963; Brawerman 1963; Sissakian *et al*. 1965; Boardman *et al*. 1965, 1966; Mehta *et al*. 1968) and amino acid activating enzymes (Bove and Raacke, 1959, Marcus, 1959; Sissakian *et al*. 1965; Hadziyev and Zalik 1970) which were found in the stroma following centrifugation of ruptured chloroplasts.

In vivo studies

Brachet et al. (1955) exposed *Acetabularia* to $^{14}CO_2$ in light for two hours and found the specific radioactivity of chloroplast protein to be higher than the microsomal proteins suggesting that chloroplast proteins were synthesised in the chloroplast. Heber (1962) exposed spinach leaves to $^{14}CO_2$ in the dark for four minutes and then illuminated them. He took samples at intervals and used a non-aqueous procedure to isolate the chloroplasts. Radioactivity was detected earlier in chloroplast proteins than the cytoplasmic proteins. Smith et al. (1961) got similar results with *Chlorella*. Parthier (1964) exposed leaves of *Nicotiana rustica* to $^{14}CO_2$ in light for 15 minutes and then kept them in the dark. The specific radioactivity of chloroplast proteins was higher than for the cytoplasmic proteins, again providing evidence for the synthesis of chloroplast proteins by the chloroplasts.

Molchanov and Bezinger (1968) performed in vivo experiments by feeding $^{14}CO_2$ to bean leaves. They found that most of the radioactivity in chloroplasts was associated with the lipoproteins.

Protein synthesis by isolated chloroplasts

Ranaletti et al. (1969) and Goffeau (1969) reported that the amino acid incorporating activity of isolated chloroplasts depended upon their remaining intact. Brief sonication of chloroplasts prior to incubation reduced the amino acid incorporating activity. Also preincubation of isolated chloroplasts at 25°

for 30 minutes reduced amino acid incorporating activity by 38% as compared with chloroplasts at 0° (Goffeau, 1969). According to Goffeau the observed effect of temperature could be due to swelling. Ranaletti et al. (1969) reported that maintaining isolated chloroplasts in hypotonic medium or resuspending and centrifugation resulted in lower activity. Generally in order to preserve the structure during isolation buffered sucrose or manitol have been used as extraction medium. Honda et al. (1962) used a complex medium which contained the protective colloids, Dextran and Ficoll for the isolation of chloroplasts.

Differential centrifugation has generally been used as a first step in chloroplast isolation. Further purification usually involved either washing or centrifugation on continuous or discontinuous sucrose density gradient.

Earlier results with isolated chloroplasts indicated amino acid incorporation to be independent of added ATP and insensitive to added Ribonuclease (Stephenson et al. 1956; Sissakian et al. 1965; Hall and Cocking 1966). According to Gnanam et al. (1969) the above observation may have been due to the chloroplasts being contaminated with bacteria when simple hypotonic buffered medium was used. They showed that "Honda" medium preserved the activity of isolated chloroplasts and also prevented bacteria from coming down along with the chloroplasts.

Spencer and Wildman (1964), Boardman et al. (1965), Spencer (1965), Bamji and Jagendorf (1966), Parenti and Margulies (1967), Goffeau (1969) and Ranalletti et al. (1969) have shown that isolated chloroplasts are active in protein synthesis. Amino acid incorporation was dependent on ATP, GTP, Mg^{++} and was sensitive to Ribonuclease and protein inhibitors suggesting that the observed activity was not due to bacteria.

Spencer and Wildman (1964) showed by cell fraction that the greatest amino acid incorporating activity was associated with the 1000 x g fraction which contained principally nuclei and chloroplasts. They also showed that nuclei did not contribute much to the amino acid incorporating activity of the 1000 x g fraction. This was shown using Triton x-100 treatment and also by separating nuclei from chloroplasts with density gradient centrifugation. Parenti and Margulies (1968), estimated the mitochondrial contribution to incorporation by bean chloroplasts as less than two percent. They also showed the contribution due to cytoplasmic ribosomes to be less than seven percent.

For amino acid incorporation studies with isolated chloroplasts, the reaction mixture generally used consists of Tris, Mg^{++}, KCl, Mercaptoethanol, ATP, PEP, Pyruvate kinase, UTP, CTP, GTP, and a mixture of nineteen cold amino acids lacking the radioactive amino acid (Spencer and Wildman, 1964). Bamji and Jagendorf (1966) used ammonium sulfate in their reaction mixture instead of amino acid mixture, as the latter was found to be

somewhat inhibitory.

Mg^{++} was required for optimal amino acid incorporating activity but an excess was inhibitory (Bamji and Jagendorf 1966; Ranalletti et al. 1969). Depending on the plant, the level of Mg^{++} varied for optimal activity.

Margulies et al. (1968) studied the inhibitory effect of ribonuclease on the amino acid incorporating activity of isolated chloroplasts from *Phaseolus vulgaris*. There was a lag period in RNase action. Preincubation of chloroplasts at 25° in the absence of ribonuclease resulted in an increased inhibitory effect of RNase on amino acid incorporating activity. They concluded that the delay was due to a change in the chloroplast membrane which was initially impermeable to RNase. They explained the lag period in RNase action as due to the presence of intact chloroplasts.

Bamji and Jagendorf (1966) studied the amino acid incorporating activity of wheat chloroplasts at different ages. They found that the activity was best when the plants were 4 or 5 days old and by the 7th day the activity dropped. They tried to correlate the loss of activity, with the disappearance of heavier components of ribosomes (polysomes).

Heber (1962), Spencer (1965), Bamji and Jagendorf (1966), Margulies et al. (1968), Goffeau (1969) and Chen and Wildman (1970) studied amino acid incorporation into different proteins. Heber (1962) found in spinach chloroplasts most of the label was in the

particulate proteins sedimenting at 20,000 x g. Also using spinach Spencer (1965) obtained 75% of the label in the particulate proteins. Bamji and Jagendorf (1966) found 50% of the label in soluble proteins in the case of wheat chloroplasts. Margulies et al. (1968) reported 50% of the label in soluble proteins with bean. Goffeau (1969) obtained about 35% of the label in the soluble proteins of *Acetabularia* chloroplasts. Using Triton x-100 and RNase treatment he showed most of the radioactivity to be associated with the membrane fragments rather than with the ribosomes. Specific radioactivity of membrane protein was higher than that of the soluble protein, suggesting that the former was actively synthesised in vitro. Margulies et al. (1968) and Goffeau (1969) found radioactivity in chloroplast structural protein which they isolated according to Criddle and Park (1964). With tobacco chloroplasts Chen and Wildman (1970) obtained about 50% of the label in the supernatant not sedimenting at 17,000 x g. Most of the radioactivity in the supernatant was associated with the 70s ribosomes and polysomes. Radioactivity in the 17,000 x g pellet was found mostly as nascent peptides attached to membrane-bound chloroplast ribosomes. No radioactivity was found in the structural protein.

In their studies Bamji and Jagendorf (1966) found that the percentage of radioactivity recovered in the soluble protein fraction increased with time of incubation. This could be taken to indicate that the insoluble component acted as a precursor of the soluble. Margulies et al. (1968) studied the proportion of

radioactivity incorporated into the particulate protein of bean chloroplasts as a function of time. The ratio of radioactivity in the particulate to total protein was constant. Also in another set of experiments in which cold leucine was fed as chase the ratio of radioactivity in proteins of soluble to insoluble remained the same. On both these lines of evidence they concluded that there was no direct evidence for soluble protein serving as a precursor for lamellar protein and vice-versa. In their experiments the lamellar fraction corresponded to 6,000 x g pellet and not 140,000 x g pellet and the supernatant obtained at 6,000 x g was referred to as soluble fraction. They noted that only a small portion of radioactivity in protein sedimented between 6,000 and 140,000 x g.

Using isolated bean chloroplasts Sissakian et al. (1963) demonstrated the incorporation of labeled amino acids into chloroplast lipids. Later Smirnov and Rodinov (1964) for isolated chloroplasts from bean, as well as pea, clover and spinach showed the incorporation of labeled amino acids into chloroplast lipids, nucleic acids and proteins. Light stimulated the incorporation of amino acids into each of these macromolecules. According to Smirnov and Rodinov (1964) incorporation of amino acids into lipids and nucleic acid was related to the process of protein synthesis.

Thus chloroplasts in vitro are capable of incorporating label into both soluble and particulate protein. On the other hand

studies with mitochondria have shown that the label is incorporated only into membrane proteins (Roodyn, 1962; Truman, 1964; Wheeldon and Lehninger, 1966; Haldan et al. 1966; Kadenbach, 1967; Neupert et al. 1967). This has been taken as evidence for a greater degree of autonomy for chloroplasts.

Protein synthesis by chloroplast ribosomes

Lyttleton (1960, 1962); Clark et al.(1963, 1964); Brawerman (1963); Sissakian et al. (1965); Boardman et al. (1965, 1966) and Mehta et al (1968) isolated ribosomes from chloroplasts with a sedimentation coefficient of nearly 70 whereas the corresponding cytoplasmic ribosomes had an S value of 80. The results are similar for the monocotyledonous and dicotyledonous species studied so far.

Boardman et al. (1966) reported studies on tobacco chloroplast ribosomes. They differed from cytoplasmic ribosomes with respect to stability. The 70S chloroplast ribosomes required a Mg^{++} concentration of 15-20mM to preserve structural integrity, whereas the cytoplasmic ribosomes were stable under lower concentrations of Mg^{++} up to 5 mM. When the Mg^{++} concentration was lowered 70S ribosomes dissociated into 50 and 35S subunits, whereas the 80S cytoplasmic ribosomes dissociated into subunits of 56 and 35S, only when the Mg^{++} was removed by dialysis with EDTA. The 70S subunits recombined to give a 70S particle when the initial Mg^{++} concentration was restored but the 80S ribosome dissociation was not reversible. Addition of Mg^{++} gave rise to larger aggregates

but not an 80S particle.

App and Jagendorf (1963); Eisenstadt and Brawerman (1963, 1964); Sissakian et al. (1965) and Boardman et al. (1966) have shown that chloroplast ribosomes are active in incoroporating amino acids into protein. In addition to the usual cofactors t-RNA, mRNA and pH5 enzymes were required for protein synthesis (Eisenstadt and Brawerman 1963, 1964). Boardman et al. (1966) used a high speed supernatant factor instead of t-RNA, mRNA and pH5 enzyme fraction. The activity of 70S ribosomes was 10 to 20 times more than for the 80S ribosomes.

The concentration of Mg^{++} required for optimal amino acid incorporating activity was different for the two classes of ribosomes. Chloroplast ribosomes required 15-20mM Mg^{++} whereas the cytoplasmic ribosomes required 5mM Mg^{++}. According to Boardman et al. (1966) this high Mg^{++} requirement by 70S ribosomes may be related to the binding of mRNA or t-RNA with the ribosomes.

Amino acid incorporation was supressed by inhibitors of protein and nucleic acid synthesis. Sissakian et al. (1965) studied amino acid incorporation in the presence of ribonuclease. RNase brought about 70% inhibition in the case of cytoplasmic ribosomes whereas in chloroplast ribosomes no appreciable inhibition was noted. However, following dialysis of chloroplast ribosomes, RNase brought about 40% inhibition. According to Sissakian et al. (1965) and Boardman et al. (1966) most of the amino acid incorporating activity was associated with the ribosome monomers.

Marcus and Feeley (1965) using wheat embryos showed that the ribosome monomer had little or no amino acid incorporating activity. For several biological systems including sea urchin eggs, wheat embryos, broad bean cotyledons and carrot root tissue, protein synthesising capacity has been linked to polysome content (Monroy and Tyler 1963; Hultin 1964; Marcus and Feeley 1966; Leaver and Key 1967; Payne and Boulter 1969). According to Marcus and Feeley (1965) polysomes formation involved amino acyl addition to a ribosome-messenger complex. The presence of RNase prevented polysome formation.

For studying polyribosomes from leaf material, it has been advantageous to use a nuclease inhibitor such as polyvinyl sulfate or bentonite to reduce RNase activity. Clark et al. (1964) used polyvinyl sulfate as a nuclease inhibitor and obtained $S_{20,w}$ values of 83, 125, 159, 186 and 202 for the ribosomes from Chinese cabbage leaves.

Bamji and Jagendorf (1966), Bartels and Weier (1967) and Mehta et al. (1968) reported the presence of polysomes in chloroplasts. Hadziyev and Zalik (1970) studied amino acid incoporating activity of chlorplast polysomes from wheat and compared them with the corresponding cytoplasmic polysomes. The former had 20-fold higher activity than the cytoplasmic polysomes. Hadziyev and Zalik (1970) reported up to penta-ribosomes in the case of chloroplasts from four day old wheat seedlings whereas in the case of seven day old only single ribosomes along with traces of di- and tri-ribosomes were obtained. Also chloroplasts from seven day old

seedlings had five-fold higher endogeneous ribonuclease activity. Hence the low amino acid incorporating activity observed with chloroplasts from seven day old wheat seedlings by Bamji and Jagendorf (1966) and Hadziyev and Zalik (1970) might have been due to the high level of RNase which degraded the polysomes.

The recent confirmation and characterization of chloroplast polysomes has stimulated studies pertaining to their site of attachment, if any, in the chloroplast. Palade (1955) has shown on the basis of electron microscopic studies the presence of polysomes attached to membranes in cytoplasm. On the other hand Weiss and Grover (1968) and Jensen (1968) showed the presence of polysomes lying free in cytoplasm. Payne and Boulter (1969) have shown the presence of free and membrane bound polysomes in the cytoplasm of bean leaves. According to them these two classes of polysomes may be involved in the synthesis of different groups of proteins. Bartels and Weier (1967) have published electron micrographs showing helically arranged polysomes lying free in the chloroplast matrix of wheat seedlings while Falk (1969) showed the presence of free and membrane-bound ribosomes in tobacco chloroplasts. It seems therefore that both types of polysomes also occur in chloroplasts. The distribution and function of these two types of polysomes in protein synthesis in chloroplasts needs to be established.

Recently Parenti-Rosina *et al*. (1969) for E.coli have shown the presence of initiating factors necessary for protein

synthesis in the subunits obtained from the polyribosomal complex. So the amino acid incorporating activity of the polyribosomal complex would depend not only on the presence of mRNA but also on the initiating factors.

Recent findings have shown chloroplast DNA to be different from nuclear DNA. (Tewari and Wildman 1966; Wells and Birnstiel 1967; and Kung and Williams 1968, 1969). It is suggested therefore that they should have an independent protein synthesising capacity. Amino acid incorporation experiments with isolated *Acetabularia* chloroplasts by Goffeau (1969) who enucleated the algae several days before the experiment, and amino acid incorporation by isolated wheat chloroplast polysomes Hadziyev and Zalik (1970) lends further support to the general concept of chloroplasts being able to synthesise their own proteins.

MATERIALS AND METHODS

Plant Material

Seed of Stewart 63 wheat (*Triticum durum* Desf.) and Kinghorn wax bean (*Phaseolus vulgaris L.*) were placed in sterilized soil and grown in a growth cabinet at 21°C under continuous illumination of 1500 foot candles and relative humidity close to 50%. Wheat leaves were harvested on the 5th day after planting whereas bean leaves were harvested on the 7th day. Prior to harvest for chloroplast isolation the seedlings were placed in darkness for 12 hours to reduce the starch content in the chloroplasts.

Isolation of Chloroplasts

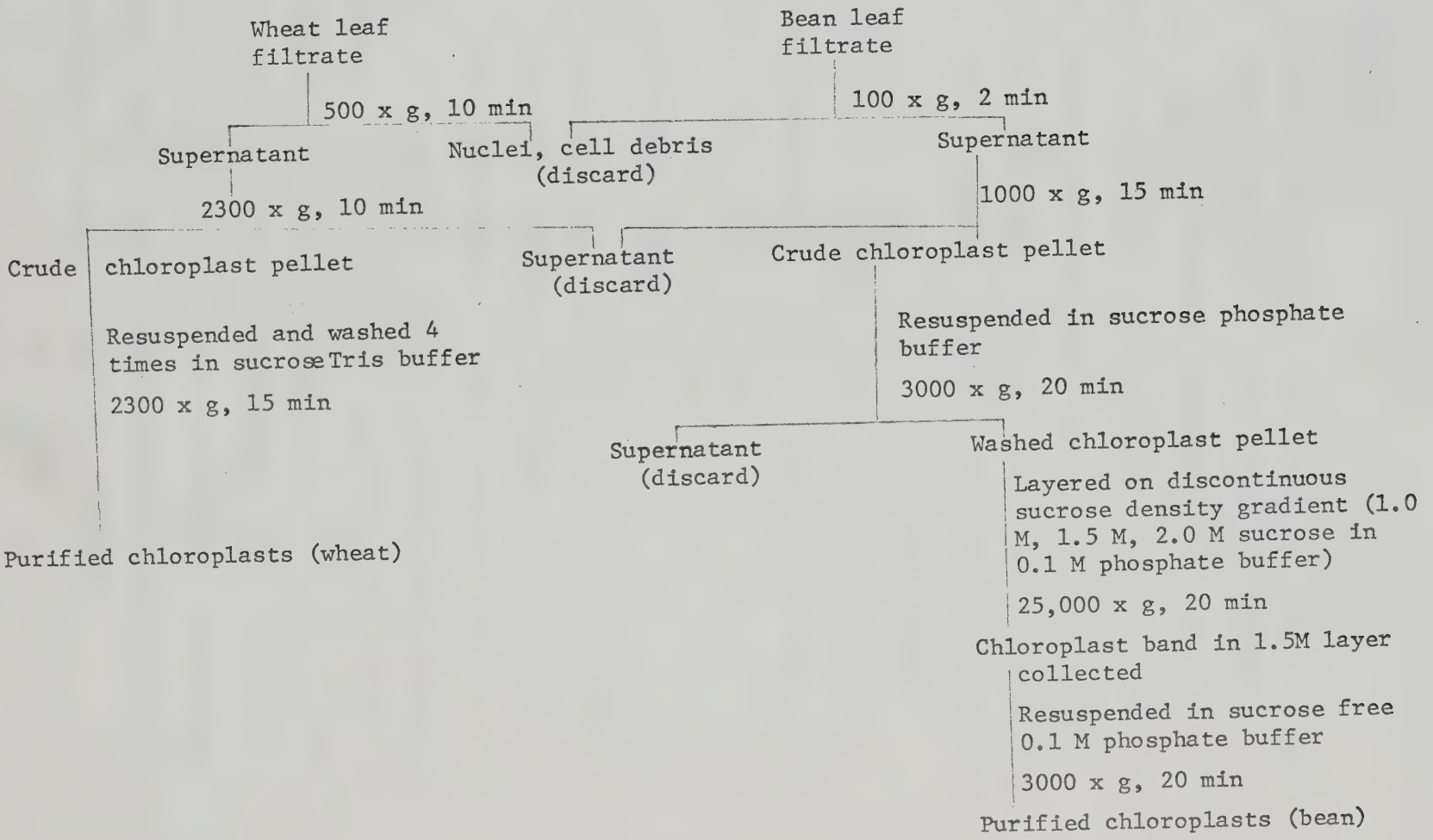
Chilled, fresh wheat leaves were ground in a mortar in a medium consisting of 0.44M sucrose; 0.1M tris (pH 7.8); 50mM KCl; 10mM $MgCl_2$ and 4 mM mercaptoethanol. The ratio of leaves to buffer was 1:2 (w/v). The grinding medium for bean leaves was 0.33M sucrose in 0.1M phosphate buffer (pH6.8); 0.01M KCl and 0.01M $MgCl_2$ (1:1.5 w/v). After filtering through cheesecloth the wheat filtrate was centrifuged at 500 x g for 5 minutes and the bean filtrate at 100 x g for 2 minutes to remove nuclei and cell debris. Wheat and bean supernatants were centrifuged at 2,300 x g for 10 minutes and 1,000 x g for 15 minutes respectively to yield the crude chloroplast pellets. All operations were at 0-5°.

Purification of Chloroplasts

The crude preparation of wheat chloroplasts was washed by suspending the crude pellet in 0.44M sucrose tris buffer and centrifuging at 2,300 x g for 15 minutes. This washing procedure was repeated four times to give the purified chloroplasts.

In the case of bean, the crude preparation was washed by suspending the crude pellet in 0.33M sucrose phosphate buffer and centrifuging at 3,000 x g for 20 minutes. The resultant pellet of chloroplasts was then purified by resuspending the pellet in 0.33M sucrose phosphate buffer and layering it on a discontinuous sucrose density gradient. The upper layer of the gradient was 1.0M sucrose, the middle one was 1.5M and the lower was 2.0M sucrose, all in 0.1M phosphate buffer. Following centrifugation at 25,000 x g for 20 minutes the chloroplast layer was retained at the interface between the 1.0M and 1.5M sucrose bands. The liquid above this layer was removed with a syringe and discarded. The chloroplast layer was then removed in a similar manner. An equal volume of sucrose-free phosphate buffer was added to the chloroplast layer and the suspension was centrifuged at 3,000 x g for 20 minutes. The pellet obtained from this centrifugition was considered as the purified chloroplast pellet. The details of the procedure are diagrammed in Fig. I.

FIGURE I Flow diagram of the isolation and purification of the chloroplasts from wheat and bean seedlings. All operations were at 0-5°C.



Nitrogen Estimation

A known weight of freeze dried purified chloroplasts was subjected to Kjeldahl digestion. Ammonia formed was determined with Nesslers reagent. Absorption measurements were read in a Beckman Model Dk-1 recording spectrophotometer at 390nm. Nesslers reagent was prepared by mixing 10gm of HgI_2, 5gm KI and 20gm NaOH in 100 ml water. It was allowed to stand for 24 hours and the clear supernatant was collected. The reagents NaOH (2N), water, Nesslers reagent and sample (hydrolysate) were mixed in the proportion 4:4:1:1 (v/v) in a total volume of 10 ml. A standard curve was prepared using ammonium sulfate. For converting percentage nitrogen into protein, a factor of 6.25 was used.

Isolation of Structural Protein

The purified chloroplasts were freed from lipid and pigments by suspending in chloroform and acetone 1:1 (v/v) and subsequent washing with acetone. Finally they were washed with ether and dried under vacuum. The lipid-free protein was suspended in 0.002M tris buffer (pH 8.5) containing 0.035M NaCl, 0.001M sodium ascorbate and a trace of $Na_2S_2O_4$. Solubilization was effected by cholate (1mg/mg protein) and deoxycholate (2mg/mg protein) and was followed by precipitation with 12-16% ammonium sulfate saturation (Criddle and Park 1964). The precipitated protein was then extracted with cold acetone to remove the detergents.

Sedimentation Studies

Sedimentation analyses of the structural protein fractions were carried out on a Spinco Model E analytical ultracentrifuge at 20°. Also the sedimentation coefficients as a function of protein concentration were determined. A series of protein concentrations ranging from 2mg/ml to 7mg/ml (dry wt basis) were employed. The medium used for sedimentation studies consisted of 1% sodium dodecylsulfate (SDS), 0.1M NaCl and 4mM 2 mercaptoethanol at pH 11.0. Sedimentation runs were made at a rotor speed of 59,780 rpm and pictures at 16 minute intervals were taken of the sedimenting boundaries using schlieren optics. For calculation of sedimenting coefficients, the photographic plates were developed and read with a microcomparator (Schachman 1957). Intrinsic sedimentation coefficients were calculated using the equation

$$S^{o}_{20w} = S_{obs} \left(\frac{\eta_t}{\eta_{20}}\right) \left(\frac{\eta}{\eta_o}\right) \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_t}\right)$$

where $\left(\frac{\eta_t}{\eta_{20}}\right)$ is the principal correction factor corresponding to the viscosity of water at t° relative to that at 20°, $\left(\frac{\eta}{\eta_o}\right)$ is the relative viscosity of the solvent to that of water and $\rho_{20,w}$ and ρ_t are the densities of water at 20° and the solvent at t°, respectively. Since the runs were made at 20°, S_{obs} values were extrapolated by zero protein concentration to give the $S^{o}_{20,w}$ values directly.

Amino Acids

Amino acid analysis of the structural protein was carried out on an amino acid analyzer according to Spackman, Stein and Moore (1958). One mg of protein was hydrolyzed in oxygen-free sealed ampules with constant boiling HCl at 110° for 20 hours. A sample of 300µl was applied to the column for analysis.

Optical Rotatory Dispersion Measurements

Optical rotatory dispersion (O.R.D.) measurements of wheat and bean chloroplast structural protein fractions in 1% SDS, 0.1M NaCl and 5mM Dithiothreitol (DTT) at pH 11.0 were obtained using a Cary Model 60 recording spectropolarimeter. The solvent was filtered through a 0.45µ Millipore filter and the protein solution through a 5.0µ filter. Readings were taken in the visible region with a 1 cm path length cell and in the ultraviolet region a 0.1 cm cell was used. The dispersion constants were calculated using the one-term Drude equation

$$[\alpha]_{\lambda} = \frac{k}{\lambda^2 - \lambda_c^2}$$

where λ_c is the dispersion constant, and $[\alpha]_{\lambda}$ is the specific rotation.

According to Yang and Doty (1957) a value of 212μm for λ_c indicates completely random coil and 282μm is equivalent to 50% helix. The percentage of helix content was calculated on this basis.

Electrophoresis

Gel electrophoresis in acetic acid was carried out according to Takayama et al. (1966). The gel system consisted of 7.5% acrylamide, 35% acetic acid and 5M urea. It was prepared by mixing stock solution A, stock solution B and N-N, N^1-N^1 tetramethyl ethylene diamine (T E M E D) in the ratio 3:1:0.02 (v/v). Stock solution A contained acrylamide (BIS) 0.16 gm, urea 12 gm, glacial acetic acid 28ml and it was made to a volume of 60ml with water. Stock solution B contained ammonium sulfate 0.30 gm, urea 12 gm, and water to a volume of 20ml. It was prepared and used the same day. The gel was polymerized at 50° for 15 minutes.

For the electrophoresis studies solubilization of the protein was effected in a medium containing phenol:acetic acid: water (1:1:1) (w/v/v) to a final protein concentration of 7mg/ml.

An aliquot of the protein solution corresponding to 200µg was applied to each tube. Both the upper and lower reservoirs of the electrophoretic apparatus were filled with 10% acetic acid. The lower electrode served as the cathode. Electrophoresis was carried out for one hour with a constant current of 5mA per tube. Amido black was used for staining and acetic acid washing was used for destaining.

Amino acid Incorporation by Isolated Chloroplasts

Chloroplast Isolation

Leaves of 4.5 day old wheat seedlings handled as described in plant materials were excised 1 cm above the soil surface and ground in a mortar in a modified Honda medium which contained 2.5% Ficoll, 5.0% Dextran, 0.25M sucrose, 8mM mercaptoethanol, 25mM tricine pH 8.2 and 1mM $MgCl_2$ according to Ranalletti *et al.* (1969). The ratio of leaves to buffer was 1:3 (w/v). After filtering through cheesecloth the filtrate was centrifuged at 500 x g for 5 minutes to remove the nuclei and cell debris. The supernatant was then centrifuged at 2,300 x g for 10 minutes to yield the crude chloroplast pellet. The chloroplast preparation was washed once and then resuspended in a sucrose free medium containing 10mM tricine pH 8.2, 1mM $MgCl_2$ and 8mM mercaptoethanol. The once washed chloroplasts were used for amino acid incorporation experiments.

Amino acid Incorporation

The reaction mixture for the assay was 0.5ml and included 0.25ml resuspending buffer containing chloroplasts together with 2.5μ moles tricine pH 8.2, 13.8μ moles KCl, 2.5μ moles $MgCl_2$, 0.3μ mole ATP, 1.25μ moles PEP, 10μg pyruvate kinase, 0.01μ mole GTP and a mixture of 0.0125μ mole of each of nineteen unlabeled amino acids omitting leucine. 1.5μC of uniformly labeled ^{14}C-L-leucine specific activity 312mCi/mM (Amersham/Searle) in 24μl was added to the reaction mixture. All reagents other than uniformly labeled ^{14}C-L-leucine, PEP and pyruvate kinase were stored frozen in small batches, thawed as required and the unused portion discarded. Incubation was for 30 minutes at 30°. At the end of the incubation period, the reaction was stopped by cooling the tubes and adding 1ml of 10% trichloracetic acid and 0.5 ml of 0.1M unlabeled leucine. Nucleic acids were extracted by hot trichloroacetic acid treatment (80° for 30 minutes) and the precipitate was collected on a 0.45μ Millipore filter. The precipitates on the filter were washed with trichloroacetic acid-leucine solution followed by water. After drying under vacuum, radioactivity was determined by scintillation counting. Liquiflour mixture contained 120 gm naphthalene, 6g PPO, 0.50 g POPOP and the volume was made to 1 litre with dioxan. 12ml of Liquiflour mixture was added to each vial for counting. For control, the chloroplast suspension was added at the end of the incubation period, just before the addition of trichloroacetic acid. A quenching

calibration curve was made using external standard. The efficiency of counting was between 65 to 75%.

Chlorophyll Determination

An aliquot of chloroplast suspension (0.5ml) was pipeted into a 25ml volumetric flask to which was added 4.5ml of water and 20ml of acetone. The mixture was centrifuged at 3,000 x g for 5 minutes. Absorption readings of the supernatant were made on a Beckman Model DK - 1 recording spectrophotometer at 645 and 663nm. Total chlorophyll as mg of chlorophyll per ml of chloroplast suspension was calculated according to Arnon (1949).

Amino acid Incorporation by Excised Wheat Leaves

Wheat seedlings were grown as described in preceding sections. Prior to harvest, the seedlings were kept in the dark for a period of 12 hours. Wheat leaves of uniform length were selected and excised 1cm above the soil surface. The leaves were harvested 3.5; 4.5 and 5.5 days after planting. Leaves weighing 5gms were placed in a 5ml beaker (1.5cm internal diameter) containing 1μC (1,200,000 cpm) of uniformly labeled ^{14}C-L-leucine in 0.5ml of water. When almost all the solution was taken up water was added and the incubation was continued for 60 minutes. Incorporation was at 19±1° in light of 1500 foot candles. To accelerate uptake a fan was directed at the leaves in the incubation cabinet. After incubation, the leaves were washed and homogenization was carried

out in the modified Honda medium. Following filtration through cheesecloth 1ml of 0.033M unlabeled leucine was added. The filtrate was centrifuged at 3,000 x g for 12 minutes to sediment the chloroplast fraction. The supernatant was centrifuged at 15,000 x g for 20 minutes to yield the mitochondrial fraction, Incorporation of ^{14}C-leucine as protein in chloroplast, mitochondria and the supernatant fractions were determined. An aliquot was taken from each fraction, the protein was precipitated with trichloroacetic acid and the precipitate was collected on Millipore filters. Counting was done as described in the preceding section.

The crude chloroplast pellet (3,000 x g fraction) was purified on a discontinuous sucrose density gradient as described earlier with bean chloroplast isolation. Following resuspension in water the purified chloroplasts were sonicated (BIOSONIK III) in the presence of ice for 2 minutes. The machine was operated at 60% of full power using a 3/8" diameter probe. The soluble and particulate chloroplast proteins were separated by centrifugation at 40,000 x g for 60 minutes and their radioactivity was measured.

Chase experiments in light

Leaves of 4.5 days old wheat seedlings were allowed to incorporate label for 15 minutes in light of 1,500 foot candles as described in the preceding section. Following this period the leaves were washed and transferred to another beaker containing unlabeled leucine (10μm/ml). The leaves were allowed to take up

unlabeled leucine for 75 minutes in light. Homogenization, isolation and purification of chloroplasts on a discontinuous sucrose density gradient were carried out as mentioned earlier. Radioactivity in the soluble and particulate proteins were determined. In another set of chase experiments in light uniformly labeled ^{14}C-leucine was fed for 90 minutes followed by unlabeled leucine for 18 hours. After purification of chloroplasts the percent radioactivity in the soluble and particulate proteins was determined. The 18 hours chase experiment was carried out with both 3.5 and 4.5 day old leaves.

Chase experiments in Dark

Feeding of labeled ^{14}C-leucine to 4.5 day old excised leaves was done as described earlier. Following 90 minutes incubation in light (1,500 ft. c) the leaves were washed. The washed leaves were transferred to another beaker containing unlabeled leucine (10μm/ml) in which they were kept for 18 hours in the dark. After purification of chloroplasts the percent radioactivity in the soluble and the particulate proteins was determined.

Isolation of Structural Protein Labeled with ^{14}C-Leucine

Leaves of 4.5 day old wheat seedlings were grown and excised as described in the previous sections. Seedlings were kept in the dark for a period of 12 hours prior to harvest. Leaves weighing 10g were placed in a 10ml beaker (2.25 cm internal

diameter) containing 2μC of uniformly labeled ^{14}C-L-leucine in 1 ml of water. Incubation was at 19±1° in light of 1500 foot candles. When almost all the label was taken up water was added and the incubation was continued for 60 minutes. After incubation the leaves were washed and homogenization was carried out in the modified Honda medium. The ratio of leaves to buffer was 1:3 (w/v). After filtering through cheesecloth 1 ml of 0.033M unlabeled leucine was added. Isolation and purification of chloroplasts on a discontinuous sucrose density gradient were carried out as described earlier. The purified chloroplasts were sonicated and the resulting suspension was centrifuged at 40,000 x g for 60 minutes.

Isolation of the labeled structural protein was essentially the same as for the unlabeled structural protein. The pellet obtained after sonication was freed from lipids and pigments by suspending in chloroform acetone 1:1 (v/v) and subsequent washing with acetone. It was then washed with ether and dried under vacuum. The lipid-free protein was suspended in 0.002M tris buffer pH 8.5 containing 0.035M NaCl, 0.001M sodium ascorbate and a trace of $Na_2S_2O_4$ was added followed by cholate (1mg/mg protein) and deoxycholate (2mg/mg protein). After 3 hours, the solution was centrifuged at 1000 x g for 5 minutes. The structural protein fraction was precipitated from the supernatant by 12-16% ammonium sulfate saturation. The precipitated protein was extracted with cold acetone to remove the detergents.

RESULTS AND DISCUSSION

Freeze dried purified chloroplasts of bean and wheat contained 32.5 and 40% protein respectively whereas a value of 50% was reported for spinach (Kirk and Tilney-Bassett, 1967). Since the chloroplasts were isolated in aqueous media there would be a loss of soluble proteins and the extent of loss would vary with the method of isolation and purification. Nevertheless the structural protein represents a considerable fraction of the chloroplast proteins. Criddle (1966) reported that 40% of the total chloroplast protein of spinach was structural protein. From preliminary studies it was estimated that the structural protein fraction of bean and wheat chloroplasts represented about 40% of the particulate proteins. Thus physicochemical characterization of this protein fraction from both species was undertaken and the results are presented in the succeeding section. It was expected that the studies of ^{14}C-leucine incorporation into chloroplast proteins would elucidate the sequence of incorporation and the degree of stability of the structural protein fraction. The second section of results and discussion deals with these studies using wheat.

I. Physicochemical Studies of Bean and Wheat Chloroplast Structural Protein

Reference was made in the literature review to the term "structural protein" which was initially given by Criddle *et al*.

(1962) to a protein fraction they isolated from beef heart mitochondria. It had no detectable enzyme activity and represented about 60% of the total mitochondrial protein. In addition it formed complexes with mitochondrial cytochromes and was viewed as a possible site for enzyme alignment. Later Criddle and Park (1964) isolated a protein component from spinach chloroplasts which they designated as chloroplast structural protein. It constituted about 40% of the total chloroplast protein and was homogeneous in the analytical ultracentrifuge. They also demonstrated its homogeneity by moving boundary electrophoresis. However Haldar et al. (1966); Tuppy et al. (1968) and Schatz and Saltzgaber (1969) have shown the heterogeneity of mitochondrial structural protein by gel electrophoresis. Also Ward and Pollak (1969) noted that microsomal structural protein was heterogeneous. In view of the apparent heterogeneity of mitochondrial and microsomal structural proteins, the term "structural protein fraction" is being used in this thesis instead of chloroplast structural protein.

The chloroplast structural protein fraction obtained from bean and wheat presumably was free of soluble proteins, since isolation and purification of chloroplasts in both species involved aqueous media. On the basis of isolation technique it should also have been freed of non-structural lamellar protein. It represented the protein fraction obtained by precipitation with ammonium sulfate at 12-16% saturation (Criddle and Park 1964). The structural protein fraction from both species was insoluble in neutral aqueous

buffer, however it could be dissolved in alkaline medium (around pH 11.0) in the presence of detergent.

For physical characterization, homogeneity of the structural protein fractions was tested on the analytical ultracentrifuge. The solvent system used for sedimentation velocity studies included 1% SDS, 0.1M NaCl and 5mM mercaptoethanol at pH 11.0. SDS was used to solubilize the protein, NaCl was employed to reduce the primary salt effect, whereas mercaptoethanol should have minimized any subunit association. A typical schlieren pattern of bean and wheat chloroplast structural protein fraction is shown in Figs. II and III. The protein fractions sedimented as a single, homogeneous peak free of lighter and heavier components and this symmetry was maintained even after 3 hours at 59,780 rpm (Figs. II and III). As a further check on homogeneity dependence of sedimentation coefficient on protein concentration was measured. There was a linear relationship. The sedimentation coefficient decreased with increasing protein concentration (Fig. IV) indicating no association or dissociation of the protein in the concentration range studied. Bean and wheat chloroplast structural protein fractions had S values of 1.2 and 1.3 respectively at zero protein concentration obtained by extrapolation (Fig. IV).

Criddle and Park (1964) reported an S value of 2.2 for spinach chloroplast structural protein. Thus S values obtained for bean and wheat differed considerably from this. The difference may be attributed to differences in species, isolation techniques

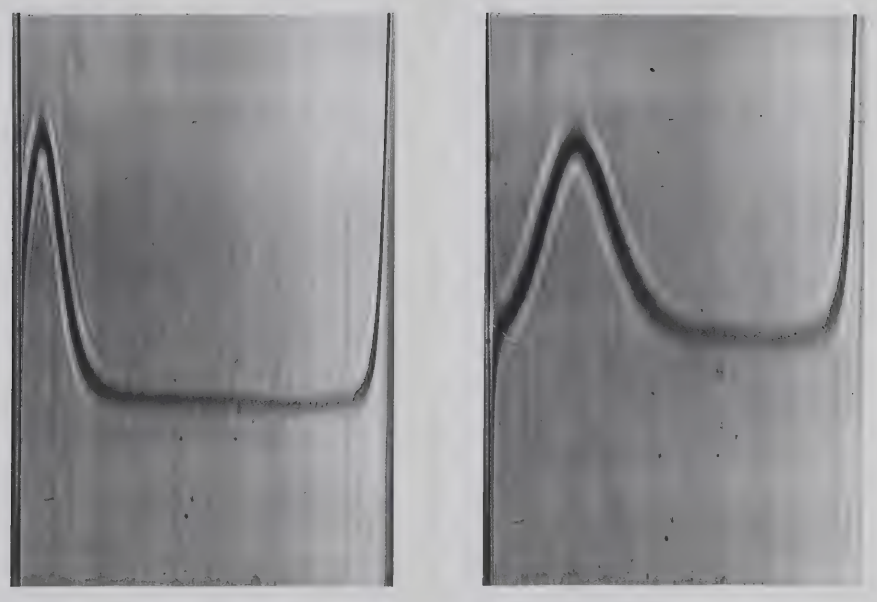


FIGURE II Sedimentation velocity pattern of wheat chloroplast structural protein fraction in 1% SDS, 0.1M NaCl, 5mM mercaptoethanol, pH 11.0 at 20°. Pictures were taken after 48 and 176 minutes at 59,780 rpm with a bar angle of 50 and 40° respectively.

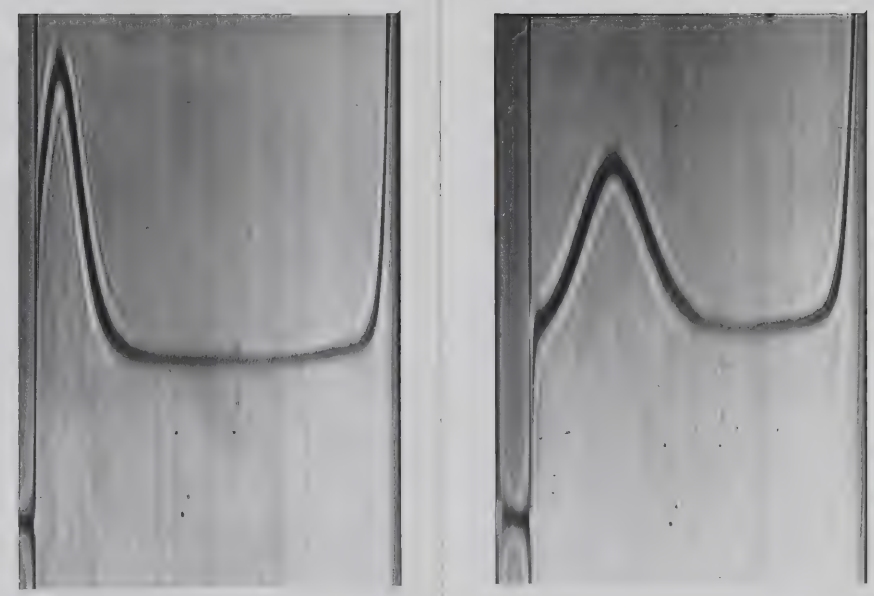


FIGURE III Sedimentation velocity pattern of bean chloroplast structural protein fraction in 1% SDS, 0.1M NaCl, 5mM mercaptoethanol, pH 11.0 at 20°. Pictures were taken after 48 and 176 minutes at 59,780 rpm with a bar angle of 45 and 40° respectively.

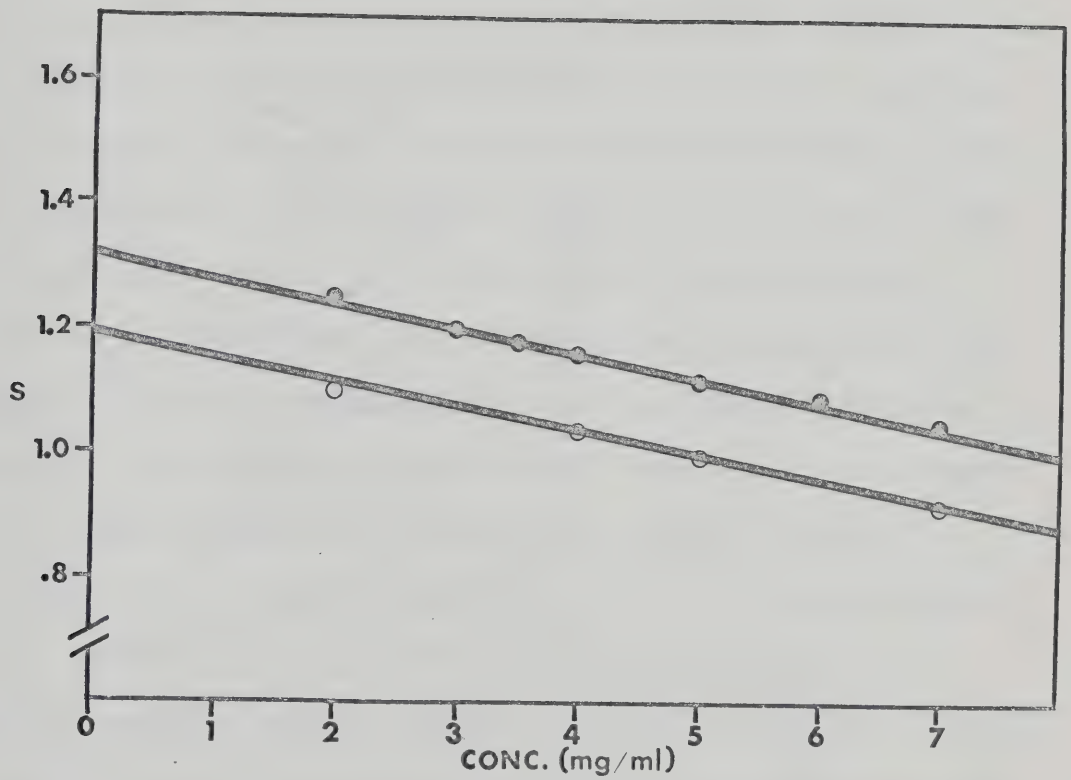


FIGURE IV Sedimentation coefficient of bean (o) and wheat (•) chloroplast structural protein fraction as a function of concentration.

and solvent systems used. During the course of isolation wheat chloroplasts were subjected to four washings and bean chloroplasts were purified on a discontinuous sucrose density gradient whereas Criddle and Park used once washed spinach chloroplasts in their studies. Purification of chloroplasts (bean and wheat) should have resulted in reducing the contamination due to other organelles. Also the solvent system for sedimentation studies differed from that used by Criddle and Park for spinach chloroplast structural protein. For spinach they used SDS (0.1 wt of protein) and 0.5M urea at pH 10.0 in 0.01M phosphate buffer. Because of the poor buffering capacity of phosphate buffer around pH 10.0 an SDS mercaptoethanol medium was used instead in these studies. Based upon the relatively low S values obtained for the structural protein fraction, it is unlikely that it contained significant amounts of non-structural lamellar protein. Although Criddle and Park approximated the molecular weight of spinach chloroplast structural protein no attempt was made to calculate the molecular weight of the protein from bean and wheat because of the presence of SDS in the solvent medium.

Amino acid composition

Table I compares the amino acid composition of the structural protein fraction from bean and wheat chloroplasts with that of other species. Values for bean and wheat are averages obtained from three replicates. Tabulated results are based on analysis of structural protein hydrolysate heated at 110° for 20

TABLE I Amino Acid Composition of Chloroplast Structural Proteins

Amino Acid	Mole %				
	Wheat	Bean	* Aceta-bularia	* Spinach	* Spinach lamellar protein
Aspartic acid	8.6	8.2	9.3	14.0	8.8
Threonine	5.5	5.8	5.6	3.7	4.7
Serine	7.7	7.1	6.0	5.5	5.7
Proline	5.9	5.4	5.3	5.1	5.9
Glutamic acid	10.4	10.2	11.0	13.1	9.2
Glycine	10.9	11.7	11.0	9.2	10.5
Alanine	10.4	9.9	11.0	10.4	9.6
Valine	6.7	6.2	7.4	7.4	6.5
Methionine	1.4	1.1	Trace	0.2	1.7
Isoleucine	4.5	4.8	5.8	6.6	5.3
Leucine	9.4	10.2	10.1	11.4	11.0
Tyrosine	1.8	2.4	1.6	0.7	3.8
Phenylalanine	4.2	5.0	5.8	0.2	6.5
Lysine	6.5	6.3	4.9	8.2	5.5
Histidine	1.7	1.7	1.5	2.1	1.4
Arginine	4.4	4.0	3.9	1.0	4.2
Cysteic acid	-	-	-	1.3	-

* Acetabularia Goffeau 1969

Spinach Criddle 1966

Spinach lamellar protein Ji et al 1968

hours in sealed ampules. No correction was made for the breakdown of amino acids during hydrolysis. The amino acids, tryptophan, cysteine and cystine were not estimated. Essentially the structural protein fraction from bean and wheat had like composition. The major differences from structural protein of spinach were in the levels of aspartate, glutamate, phenylalanine and arginine. However the values of bean and wheat were similar to chloroplast structural protein from *Acetabularia* as can be seen from the table. The amino acid composition was similar to spinach lamellar protein. The relatively high levels of non-polar amino acids in the chloroplast structural protein fraction could give rise to extensive hydrophobic regions which might account for protein stability (Green et al. 1961).

Protein conformation

Conformation of the structural protein fraction was studied by ORD using a solvent system similar to that employed for sedimentation analysis. However in ORD measurements, dithiothreitol was used in place of mercaptoethanol, as the latter was found to absorb appreciably in the region of the protein conformational cotton trough (Wolfe and Kay 1967). The one-term Drude equation was used to calculate the dispersion constants. There was a linear relationship when optical rotation was plotted against optical rotation times the square of wave lengths (Figs. V and VI). In the visible region the constant was 223nm for bean and 245nm for

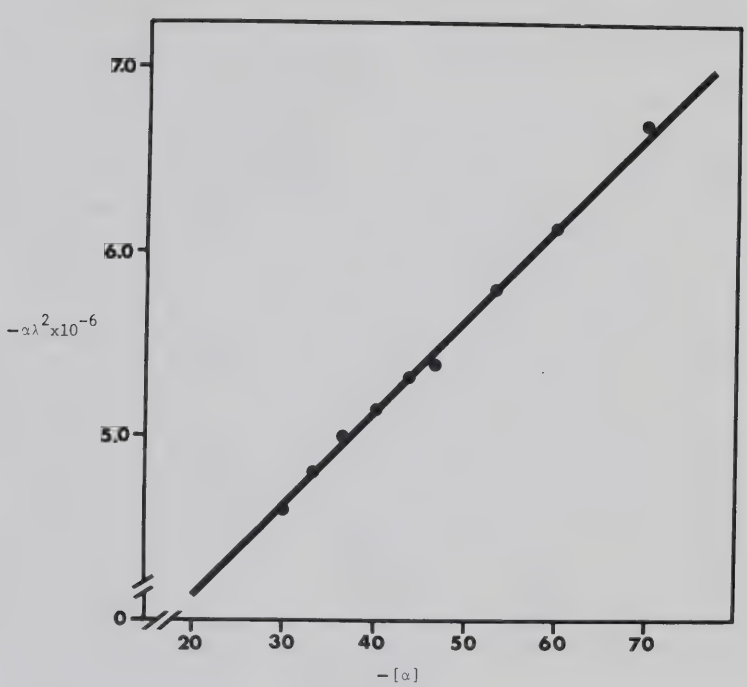


FIGURE V Graphical representation of One-term Drude equation as applied to bean chloroplast structural protein. The solvent system included 1% SDS, 0.1M NaCl, 5mM DTT pH 11.0. Readings were taken in the range 300-420nm with a Cary model 60 recording spectropolarimeter using 1-cm path length cell.

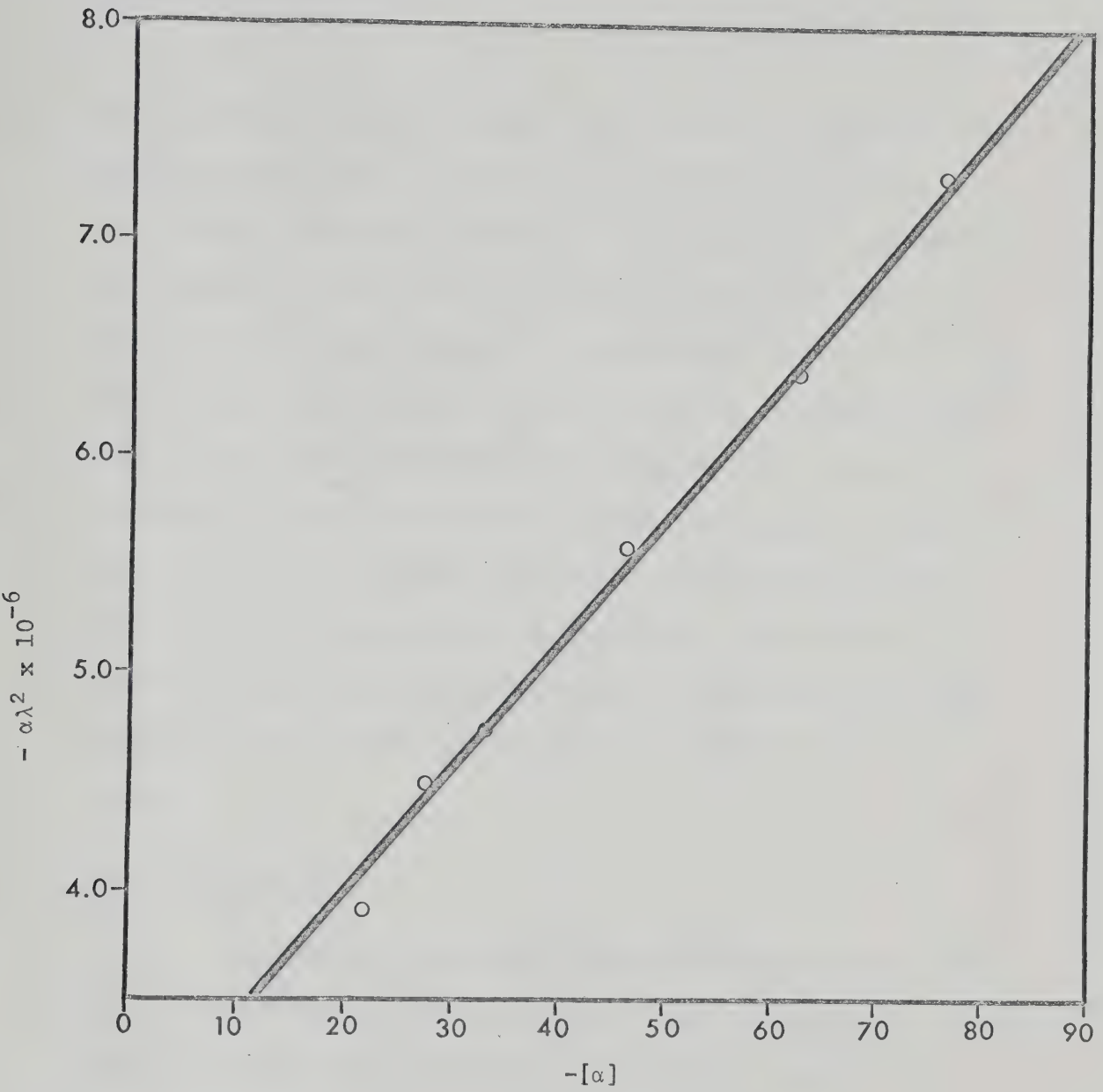


FIGURE VI Graphical representation of One-term Drude equation as applied to wheat chloroplast structural protein. The solvent system included 1% SDS, 0.1 M NaCl, 5mM DTT pH 11.0. Readings were taken in the range 300-420nm with a Cary model 60 recording spectropolarimeter using 1-cm path length cell.

wheat, indicating α-helix content of 8% and 23% respectively. In the ultraviolet region a cotton trough at 233nm was obtained for both species, additional evidence for the presence of α-helix in these proteins. Also Stein and Fleischer (1967) have shown the presence of α-helical structure in mitochondrial structural protein. However the values obtained with bean and wheat chloroplast structural protein fraction should not be taken as a true estimate of the content of α-helical structure, since the protein is likely to lose a part of its secondary structure in the presence of low hydrogen ion concentration (ph 11.0) and SDS. Nevertheless the results have been taken as evidence for the presence of α-helical structure in the chloroplast structural protein fraction of both species.

Gel Electrophoresis

Two proteins could have nearly the same molecular weight and still be different with respect to charge. Sedimentation studies could not detect such a difference, if present. Therefore, gel electrophoresis of the proteins was carried out as a further test of homogeneity. Five bands were obtained for bean and 2 bands in the case of wheat (Fig. VII). Hence, they were heterogeneous at least with respect to charge. Moreover in this respect bean and wheat differed from each other and from spinach for which Criddle (1966) reported a single boundary using boundary electrophoresis.

In addition to the difference in species, the apparent

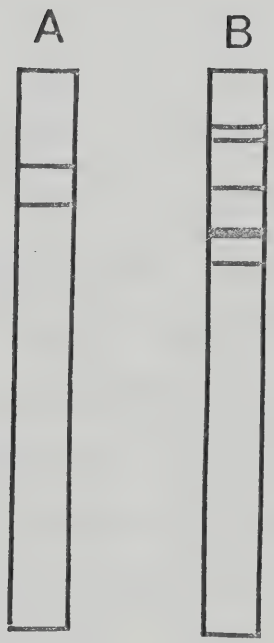


FIGURE VII Diagrams of polyacrylamide gel electrophoretic profiles of chloroplast structural protein fractions. Electrophoresis carried out in 10% acetic acid with a constant current of 5mA per gel. Cathode at bottom; application point at top. A. Wheat. B. Bean.

homogeneity of spinach chloroplast structural protein by electrophoresis may have been due to differences in the methods and technique used. The solvent system used to solubilize the proteins from bean and wheat was phenol, acetic acid and water in the ratio of 1:1:1 (w/v/v) whereas Criddle used 0.1M tris, 0.05% SDS and 0.5M urea at pH 8.5 for solubilizing spinach cloroplast structural protein. Gel electrophoresis for bean and wheat was carried out in 10% acetic acid while boundary electrophoresis for spinach by Criddle was at pH 8.5. Because of the difference in the pH values used, the net charge on the proteins would also have been different and this could account for the observed effect. Mitochondrial and microsomal structural proteins have been shown to be heterogeneous by gel electrophoresis (Haldar et al. 1966; Tuppy et al. 1968; Schatz and Saltzgaber 1969; Lejsek and Lusena 1969 and Ward and Pollak 1969). On the basis of the principle involved, gel electrophoresis might be expected to give better resolution than boundary electrophoresis.

II. ^{14}C-Leucine Incoporation into Chloroplast Proteins of Wheat

(a) Amino acid incorporation by isolated chloroplasts

Amino acid incorporating activity of isolated wheat chloroplasts was shown to depend on the age of the plant (Bamji and Jagendorf, 1966 and Hadziyev and Zalik, 1970). Hence leaves from 4.5 day old wheat seedlings were used for chloroplast isolation. A modified Honda medium containing 1mM Mg^{++} was used for chloroplast

extraction (Ranalletti et al. 1969) and once washed chloroplasts were used for incorporation experiments.

Table II shows the ability of isolated chloroplasts to incorporate amino acids into protein. The values reported are based on feeding for 30 minutes at 30°C. For control the chloroplast suspension was added at the end of the incubation period. This was intended to serve as a check for bacterial contaminants in the reaction mixture reagents. According to Gnanam et al. (1969) Honda medium prevented a major part of the bacteria from being collected together with the chloroplasts. Their conclusion was based on the requirement of ATP for amino acid incorporation by such chloroplast preparations and their sensitivity to ribonuclease. Modified Honda medium of Gnanam et al. (1969) was used for chloroplast isolation and the observed increased activity on the addition of ATP and GTP further supports the view that the observed incorporation is due to chloroplasts.

The incorporation of 8000 cpm per mg chlorophyll in 30 minutes is higher than that reported for isolated wheat chloroplasts by Hadziyev and Zalik (1970). However, they used Honda medium in tris buffer (pH 7.8) for chloroplast isolation and fed a [^{14}C] amino acid mixture. In this study isolation was in Honda medium in tricine buffer and labeled leucine was fed. Also they used 4 day old wheat seedlings of another variety, instead of 4.5 day old seedlings and the amount of radioactivity fed and other conditions

TABLE II Incorporation of ^{14}C-Leucine by Isolated Chloroplasts from 4.5 day old Wheat Seedlings

The complete reaction mixture contained in μ moles: tricine buffer (pH 8.2) 2.5; KCl, 13.8; $MgCl_2$, 2.5, ATP, 0.3; GTP, 0.01; PEP, 1.25; each of nineteen amino acids (lacking leucine) 0.0125; pyruvate kinase, 10 μg; 0.25 ml chloroplast suspension (80-100 μg chlorophyll) in a volume of 0.5 ml plus 1.5 μC [^{14}C] leucine (specific activity, 312 mC/mmole).

Reaction Mixture	Incorporation* cpm/mg Chlorophyll
complete	8000
-ATP -GTP	1500

* In this and all subsequent tables incorporation is expressed as hot trichloroacetic acid insoluble radioactivity.

The data are averages of three experiements.

varied. The observed difference in amino acid incorporation may have been due to a combination or any one of these factors. Ranalletti et al. (1969) have reported for isolated wheat chloroplasts a value of 40,000 cpm per mg chlorophyll for 60 minute incubation. They used a [^{14}C] amino acid mixture supplemented with ammonium sulfate instead of ^{14}C-leucine plus unlabeled amino acids and the seedlings were grown under 15 hour day light, whereas in this study the seedlings were grown under continuous illumination.

When isolated chloroplasts are fed, the reaction mixture normally has a chloroplast suspension containing about 100 µg chlorophyll since this gives an optimum level of incorporation. This would correspond to a value of only 4000 cpm even if the incorporation rate were 40,000 cpm per mg chlorophyll. Thus after purification and separation into soluble, particulate and structural proteins, the amount of incorporation in individual fractions would be extremely low. Since in this study maximum incorporation by isolated chloroplasts was 8000 cpm per mg chlorophyll1 whereas when excised leaves were fed in the absence of any cofactors, the incorporation of label into chloroplast proteins was much higher the remaining studies were carried out using excised leaves. Feeding label to excised leaves rather than to a purified chloroplast preparation has the inherent disadvantage that the apparent protein synthesis in the chloroplasts might not be independent of other organelles. However, it also has the advantage of minimizing bacterial involvement.

(b) Amino acid incorporation by excised wheat leaves

Table III shows the distribution of radioactivity among different centrifugal fractions. It can be seen from the table that the leaves of 4.5 day old seedlings were the most active in incorporating amino acids into protein. This is in agreement with the results obtained with isolated chloroplasts (Bamji and Jagendorf 1966 and Hadziyev and Zalik, 1970). For the same period of incubation and amount of label fed, leaves from 5.5 day old seedlings were only 50% as efficient as those from 4.5 day old seedlings. Both 4.5 and 5.5 day old leaves took up almost all the radioactive solution in about 15 minutes, whereas 3.5 day old leaves required 30 to 40 minutes. Proteins of the chloroplast fractions were labeled. The label in chloroplast, mitochondrial, and the supernatant fractions represented 94 to 96% of the label in the crude homogenate protein.

In order to study the distribution of radioactivity between the soluble and the particulate proteins, the chloroplast fraction (3000 x g) from 3.5, 4.5 and 5.5 day old seedlings was purified on a discontinuous sucrose gradient. The purified chloroplasts were then sonicated, fractionated, and the radioactivity in the soluble and the particulate proteins was determined.

The results obtained are shown in Table IV. Distribution of radioactivity between the soluble and the particulate proteins of chloroplasts changed with seedling age. With chloroplasts from 3.5 day old seedlings about 40% of the label was in

TABLE III Radioactivity in Proteins in Different Fractions from Excised Wheat Leaves (5 gm) fed ^{14}C-Leucine in Light (60 minutes at 1500 ft. c.).

Days after planting	Fraction	Incorporation cpm/fraction	% of Total
	Leaf homogenate	195,000	100
	3,000 x g Pellet	20,000	10
3.5	15,000 x g Pellet	18,000	9
	Supernatant	150,000	77
	Leaf homogenate	370,000	100
	3,000 x g Pellet	38.000	10
4.5	15,000 x g Pellet	40,000	11
	Supernatant	275,000	74
	Leaf homogenate	165,000	100
	3,000 x g Pellet	18,000	10
5.5	15,000 x g Pellet	19,000	11
	Supernatant	120,000	73

The data in this and subsequent tables are average of three separate experiments carried out in triplicate.

TABLE IV Radioactivity in Soluble and Particulate Proteins of Chloroplasts.

Excised wheat leaves (5 gm) were fed ^{14}C-leucine for 60 minutes in light following which the chloroplasts were isolated and purified on a discontinuous sucrose gradient.

Days after planting	Fraction	Incorporation cpm/fraction	% of Total
3.5	Chloroplasts layered on the gradient	19,000	100
	Chloroplasts recovered from the gradient	11,400	60
	40,000 x g pellet (particulate proteins)	7,000	62*
	40,000 x g supernatant (soluble proteins)	4,200	38*
4.5	Chloroplast layered on the gradient	35,000	100
	Chloroplasts recovered from the gradient	22,000	62
	40,000 x g pellet	16,300	76*
	40,000 x g supernatant	5,100	24*
5.5	Chloroplasts layered on the gradient	17,000	100
	Chloroplasts recovered from the gradient	10,300	60
	40,000 x g pellet	7,800	78*
	40,000 x g supernatant	2,200	22*

* The radioactivity is expressed as a percent of radioactivity in the chloroplasts recovered from the gradient.

the soluble proteins not sedimenting at 40,000 x g whereas in the case of chloroplasts from 4.5 day old seedlings about 25% was in the soluble proteins. There was no significant difference in the distribution of radioactivity between the soluble and the particulate proteins in the case of chloroplasts from 4.5 and 5.5 day old seedlings. The distribution of the label did not change after 4.5 days. This may be an indication that chloroplast development is complete at this stage. In the case of chloroplasts from 3.5 day old seedlings there seemed to be a preferential synthesis of soluble proteins. The observed effect of age on the distribution of the label between the soluble and the particulate proteins probably depicted the synthetic capabilities of the chloroplasts *in vivo* at different stages of development.

Bamji and Jagendorf (1966) using isolated wheat chloroplasts for amino acid incorporation studies obtained about 50% of the label in the particulate proteins. With isolated chloroplasts the crude chloroplast preparation, once washed, has usually been used, as there might be a loss in activity on purification. In most instances the distribution of label in chloroplast proteins is then estimated without prior purification of the chloroplasts. Thus when isolated chloroplasts are used account must be taken of the contributions due to various contaminants (broken chloroplasts, nuclei, mitochondria, cytoplasmic ribosomes). To avoid this, after feeding the excised leaves, the chloroplasts were isolated

and purified on a discontinuous sucrose density gradient prior to separation of the soluble and the particulate proteins. Although similar purification could be carried out on isolated chlorplasts after feeding, the incorporation by isolated chloroplasts was low (Table II). There was relatively more active synthesis of particulate proteins into chloroplasts, in leaves from 4.5 day old than from 3.5 day old seedlings.

Proteins not sedimenting at 40,000 x g were referred to as the soluble proteins and this supernatant will include in addition to the soluble proteins, intact ribosomes and ribosome fragments. The 40,000 x g centrifugation was used to insure that ribosomes would not sediment with the membrane fragments which represent the particulate proteins. The use of higher centrifugal forces >100,000 x g might result in some of the ribosomes sedimenting along with the particulate proteins.

(c) Chase experiments

Excised wheat leaves from 4.5 day old seedlings were fed ^{14}C-leucine for 15 minutes and then they were allowed to take up unlabeled leucine for 75 minutes. After a total period of 90 minutes the chloroplasts were isolated and purified. In another experiment the chloroplasts were isolated and purified after feeding ^{14}C-leucine for 15 minutes. As another control excised leaves were allowed to take up ^{14}C-leucine for 90 minutes following which the chloroplasts were isolated.

Table V shows the results obtained with the chase experiments. If the soluble protein were to serve as an intermediate in the formation of particulate proteins then there should be a transfer of label from the protein of the soluble fraction to the particulate fraction during the chase. It can be seen from the table that there was no change in the distribution of the label between the soluble and the particulate proteins during the 75 minutes chase. By comparing with the results of the 15 minute control experiment it was concluded that the incorporation was stopped soon after the introduction of unlabeled leucine. However when feeding was continued for 90 minutes without any unlabeled leucine, incorporation continued showing that the system was actively incorporating label during this period.

In another experiment excised leaves were fed ^{14}C-leucine for 90 minutes following which they were allowed to take up unlabeled leucine for 18 hours. Table VI shows the results obtained. The percent radioactivity in the soluble and the particulate proteins did not change during the 18 hour chase. From the results obtained with chase experiments it is concluded that the soluble proteins as a whole do not serve as a precursor for the particulate proteins. Since there was a significant difference in the distribution of the label in chloroplasts from leaves of 3.5 and 4.5 day old seedlings, an 18 hour chase was also carried out with 3.5 day old leaves. The results are included in Table VI.

TABLE V Radioactivity in Soluble and Particulate Proteins of Chloroplasts.

Five grams of leaves excised from 4.5 day old wheat seedlings were fed for 15 minutes with ^{14}C-leucine followed by 75 minutes chase in light. The chloroplasts were isolated and purified on a discontinuous sucrose gradient.

Time min ^{14}C-leucine	Time min ^{12}C-leucine	Fraction	Incorporation cpm/fraction	% of Total
15	75	Chloroplasts layered on the gradient	15,000	100
		Chloroplasts recovered from the gradient	9,500	63
		40,000 x g pellet (particulate proteins)	7,200	77*
		40,000 x g supernatant (soluble proteins)	2,100	23*
15	-	Chloroplasts layered on the gradient	12,000	100
		Chloroplasts recovered from the gradient	7,700	64
		40,000 x g pellet	5,800	78*
		40,000 x g supernatant	1,600	22*
90	-	Chloroplasts layered on the gradient	49,000	100
		Chloroplasts recovered from the gradient	30,300	62
		40,000 x g pellet	23,500	79*
		40,000 x g supernatant	6,100	21*

* The radioactivity is expressed as a percent of radioactivity in the chloroplasts recovered from the gradient.

TABLE VI Radioactivity in Soluble and Particulate Proteins of Wheat Chloroplasts.

Excised leaves (5 gm) were fed for 90 minutes in light with ^{14}C-leucine followed by an 18 hour chase in light or dark. The chloroplasts were isolated and purified on a discontinuous sucrose gradient.

Days after planting	Fraction	Incorporation* cpm/fraction	% of Total
3.5 chase in light	Chloroplasts layered on the gradient	30,600	100
	Chloroplasts recovered from the gradient	15,800	50
	40,000 x g pellet (particulate proteins)	11,100	72*
	40,000 x g supernatant (soluble proteins)	4,300	28*
4.5 chase in light	Chloroplasts layered on the gradient	46,000	100
	Chloroplasts recovered from the gradient	25,200	55
	40,000 x g pellet	18,900	78*
	40,000 x g supernatant	5,600	22*
4.5 chase in dark	Chloroplasts layered on the gradient	43,000	100
	Chloroplasts recovered from the gradient	21,800	50
	40 x g pellet	18,800	87*
	40 x g supernatant	2,700	13*

* The radioactivity is expressed as a percent of radioactivity in the chloroplasts recovered from the gradient.

When leaves from 3.5 day old seedlings were fed ^{14}C-leucine for 60 minutes, 38% of the label went into the soluble proteins (Table IV). However following 18 hour chase only 28% was found to be associated with the soluble proteins. This might have been expected since chloroplasts from 4.5 day old leaves had 23% of radioactivity associated with the soluble proteins. This change in relative amounts of label could have been due to a transfer of label from the soluble protein to particulate protein. Alternatively during the 18 hour chase newly synthesised chloroplast proteins might have been metabolized at different rates and the results might reflect different degrees of stability.

The percentage of label in the soluble fraction did not change during 18 hour chase in light in the case of chloroplasts from 4.5 day old leaves. It is likely that in the dark soluble and particulate proteins are metabolized at different rates. Consequently an 18 hour chase in the dark was carried out using leaves from 4.5 day old seedlings. The results are given in Table VI. Following 18 hour chase in the dark only 13% radioactivity was found to be associated with the soluble proteins, whereas following an 18 hour chase in light 22% of radioactivity was found in the soluble proteins. Conversely the relative amount of label in the membrane proteins changed from 78% in light to 87% in the dark. These changes were taken as evidence for greater stability of the membrane proteins.

(d) Isolation of labeled structural protein

Since the structural protein fraction constitutes a major component of chloroplast membrane proteins an attempt was made to isolate labeled structural protein. After feeding leaves from 4.5 day old seedlings with ^{14}C-leucine, the structural protein fraction was isolated. Table VII shows the radioactivities associated with different fractions during the isolation of the structural protein. Nearly 10% of the label in the purified wheat chloroplast was found to be associated with the strucutural protein fraction. About 75% of the radioactivity in the purified chloroplasts was found in the particulate proteins which is in accord with the earlier findings with excised leaves from 4.5 day old seedlings. Also to be noted is the fact that about 50% of the label in the particulate protein was recovered in the fraction soluble in cholate and deoxycholate from which the structural protein fraction containing about 25% of the label was isolated by ammonium sulfate precipitation.

Margulies and Parenti (1968) and Goffeau (1969) have also isolated labeled structural protein from bean and *Acetabularia* chloroplasts respectively. However, the structural protein fraction of Margulies and Parenti (1968) was isolated from a crude bean chloroplast preparation, whereas in the present study the chloroplasts were purified on a discontinuous sucrose gradient prior to the isolation of structural proteins.

TABLE VII Radioactivity in the Chloroplast Structural Protein Fraction of Wheat.

Ten grams of leaves excised from 4.5 day old seedlings were fed ^{14}C-leucine for sixty minutes in light. The chloroplasts were purified on a discontinuous sucrose gradient. Following sonication and removal of lipids, the structural protein fraction was isolated using ammonium sulfate.

No.	Fraction	Radioactivity Incorporated	
		cpm/fraction	% of other fraction
1	Purified chloroplasts	24,000	
2	Water soluble proteins	5,100	21 of 1
3	Particulate proteins	18,500	77 of 1
4	Fraction soluble in cholate and deoxycholate	8,800	48 of 3
5	Fraction insoluble in cholate and deoxycholate	6,200	34 of 3
6	Structural protein fraction	2,100	24 of 4

SUMMARY AND CONCLUSION

Although the results of individual experiments were discussed previously an attempt will be made here to summarize the findings of various aspects of the study.

Mitochondrial and microsomal structural proteins have been shown to be heterogeneous by gel electrophoresis (Haldar et al., 1966; Lenaz et al., 1968; Tuppy et al., 1968 and Ward and Pollak, 1969). Since the chloroplast structural proteins from bean and wheat were also found to be heterogeneous the term structural protein fraction has been used generally in this thesis. Sedimentation velocity studies showed homogeneous profiles in both species. S values of 1.2 and 1.3 obtained for bean and wheat chloroplast structural protein fraction differed from that of spinach for which Criddle and Park (1964) reported 2.2S. The observed difference in S values may be attributed to differences in species, isolation techniques and solvent systems used. The sedimentation coefficient decreased with increasing protein concentration indicating no association or dissociation of the protein in the concentration range studied.

The amino acid composition of bean and wheat chloroplast structural protein fraction was similar. The values for bean and wheat were also similar to chloroplast structural protein from *Acetabularia*. However bean and wheat differed from spinach chloroplast structural protein in the levels of aspartate, glutamate,

phenylalanine and arginine. The presence of relatively high levels of nonpolar amino acids in the chloroplast structural protein fraction could give rise to extensive hydrophobic regions which might account for protein stability (Green et al.,1961).

Optical rotatory dispersion measurements indicated the presence of α-helix in the structural protein fraction of both bean and wheat, with values of 8 and 23% respectively. In the ultraviolet region a cotton trough at 233mμ characteristic of proteins was obtained for both species.

On polyacrylamide gel electrophoresis 5 bands were obtained for bean and 2 in the case of wheat. Hence the structural protein fractions from bean and wheat were different and not homogeneous.

^{14}C-leucine incorporation into chloroplast proteins

Isolated chloroplasts from wheat were active in incorporating leucine into protein. As the incorporation(8000 cpm per mg chlorophyll) obtained was low for studying the radioactivity in the soluble and the particulate proteins, excised leaves were used for this purpose.

When wheat leaves excised from 3.5, 4.5 and 5.5 day old seedlings were fed with ^{14}C-leucine, label was incorporated into the chloroplast proteins. Chloroplasts were purified on a discontinuous sucrose density gradient prior to separation of the soluble and the particulate proteins. Distribution of radioactivity between the

soluble and the particulate proteins of chloroplasts changed with seedling age. With chloroplasts from 3.5 day old seedlings about 40% of the label was in the soluble proteins whereas in the case of chloroplasts from 4.5 and 5.5 day old seedlings about 25% was in the soluble proteins. Radioactivity present in the soluble and the particulate proteins at different ages might be related with chloroplast development. Proteins not sedimenting at 40,000 x g were considered as soluble proteins. The 40,000 x g centrifugation was used to avoid the sedimentation of ribosomes along with the particulate proteins.

During 75 minute and 18 hour chase experiments in light with excised leaves from 4.5 day old seedlings there was no change in the distribution of the label. Hence the soluble protein as a whole did not appear to serve as a precursor of particulate proteins. However during 18 hours chase in light with excised leaves from 3.5 day old seedlings the radioactivity in the soluble proteins changed from 38 to 28% which might be taken as an indication of transfer of label. Alternatively the newly synthesised chloroplast proteins might have been metabolized at different rates and the results might reflect different degrees of stability. With excised leaves from 4.5 day old seedlings, following 18 hour chase in the dark only 13% radioactivity was found to be associated with the soluble proteins whereas following an 18 hour chase in light 22% of the radioactivity was found in the soluble proteins. These changes were taken as evidence for greater stability of the membrane

proteins, which is in accordance with their possible role in chloroplast structure and organization. Nearly 10% of the label in the purified chloroplast was present in the structural protein fraction.

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